	AA				
1	1	${\tt MADEAALALQPGGSPSAAGADREAASSPAGEPLRKRPRRDGPGLERSPGEPGGAAPEREVPAAARGCPGAAAAALWREAEAEAAAAGGEQEAQATAAAGEGDNGPGLQGPSRE$	113	Q96EB6	SIR1_HUMAN
2	1	MA	2	Q8IXJ6	SIR2_HUMAN
3	1		59 0	Q9NTG7 Q9Y6E7	SIR3_HUMAN
5	1		0	Q918E7	SIR4_HUMAN SIR5_HUMAN
6	1		0	Q8N6T7	SIR6_HUMAN
7	î	MAAGGLSRSERKA	13	Q9NRC8	SIR7_HUMAN
	114	PPLADNLYDEDDDDEGEEEEAAAAAIGYRDNLLFGDEIITNGFHSCESDEEDRASHASSSDWTPRPRIGPTFYQQHLMIGTDPRTILKDLLPETIPPPELDDMTLWQIVINILSEPPK	233	Q96EB6	SIR1_HUMAN
	3 60	EPDPSHPLETQ···AG··········K······VQEAQDSDSDSEGGAAGGEADMDFLRNLFSQ······TLSLGSQ PLDPARPLORP···PRPE·········VPRAFR········SVVGSG	54 115	Q8IXJ6 Q9NTG7	SIR2_HUMAN SIR3 HUMAN
	1	PEUPARFLURP PRPE VYNAFK RUPAGAPFSFF331KGGRK13578VA 33VVSSG MKMSFAL TFRSAKGRWI AIPSOPCSKASI GLFVPASP GFFVASP	37	Q9Y6E7	SIR4_HUMAN
	1		31	Q9NXA8	SIR5 HUMAN
	1		30	Q8N6T7	SIR6_HUMAN
	14	-AERVRRLREEQQRERLRQVSRILRKAAAE-RSAEEGRLLAESADLVTELQGRSRRREGLKRRQEEVCDDPEEL	85	Q9NRC8	SIR7_HUMAN
		.:: ***:*: :			
	234	RKKRKDINTIEDAVKLLQECKKIIVLT WYVSCGIPDFRSRD-GIYARLAVDFPDLPDPDAMFDIEYFRKDPRPFFKFAKEIYPGGFQPSLCHKFIALSDKEGKLLRNYT	344	Q96EB6	SIR1 HUMAN
	55	KERLLDELTLEGVARYMOSERCRRVICLVGAGISTSAGIPDFRSPSTGLYDNLEKYHLPYPEAIFEISYFKKHPEPFFALAKELYPGOFKPTICHYFMRLLKDKGLLLRCYT	166	Q8IXJ6	SIR2 HUMAN
	116	GSSDKGKLSLQDVAELIRARACQRVVVMV <mark>GAG</mark> ISTPSGIPDFRSPGSGLYSNLQQYDLPYPEAIFELPFFFHNPKPFFTLAKELYPGNYKPNVTHYFLRLLHDKGLLLRLYT	227	Q9NTG7	SIR3_HUMAN
	38	PLDPEKVKELQRFITLSKRLLVMT ISTESGIPDYRSEKVGLYARTDRRPIQHGDFVRSA <mark>M ROBYMSAM</mark> NFVGWPQFSSHQPNPAHWALSTWEKLGKLYWLVT	142	Q9Y6E7	SIR4_HUMAN
	32	-KMARPSSSMADFRKFFAKAKHIVIISAAAVSAESGVPTFRGAGG-YWRKWQAQDLATPLAFAHNPSRVWEFYHYRREVMGSKEPNAGHRAIAECETRLGKQGRRVVVIT	139	Q9NXA8	SIR5_HUMAN
	31 86	ERKVWELARLVWQSSSVYFHT G-GISTASGIPDFRGPH-GVWTMEERGLAPKFDTTFESARPTQTHMALVQLERVGLLRFLVSRGKVRELASAVRNAKYLVYYTG-GISTAASIPDYRGPN-GVWTLLQKGRSVA-DLSEAEPTLTHMSITRLHEQKLVQHVVS	112 166	Q8N6T7 Q9NRC8	SIR6_HUMAN SIR7_HUMAN
	00	Sirtuin Start Site Helical Region of Interest	100	QUANCO	31K/_HUHAN
		** * *, : * : : * * * * : : : : : : : :			
	345		430	Q96EB6	SIR1_HUMAN
	167	QNIDTLERIAGLEQEDLYEA <mark>H</mark> GTFYTSHCVSASCRHEYPLSWMKEKIFSEVTPKCEDCQSLVKPDIVFFGESLPARFFSCMQSDF	251	Q8IXJ6	SIR2_HUMAN
	228	QNIDGLERVSGIPASKLVEAHGTFASATCTVCQRPFPGEDIRADVMADRVPRCPVCTGVVKPDIVFFGEPLPQRFLLH-VVDF QNVDALHTKAGSRRLTELHGCMDRVLCLDCGEOTPRGVLQERFQVLNPTWSAEAHGLAPDGDVFLSEEQVRSFQVPTCVQCGGHLKPDVVFFGDTVNPDKVDFVHKRV	309 250	Q9NTG7	SIR3_HUMAN
	143	UNVDALHIKAGSKKLIELHGURKYLLI-JUGGGUIPKGYLUEKFUVLKFINSACANGLAPDGUVFLSEGUYKSTUVFLVUGGHLKPUVFFGENLDYK-VUVFKKK ONIDELHKAGTKNLIELHGSLFKTRCTSCGVYAGNYKSPICPALSGKG-APPEGTO-DASIPYEKPECEEAGGGLLRPHVYFGENLDPAILEEVDREL	239	Q9Y6E7 Q9NXA8	SIR4_HUMAN SIR5_HUMAN
	113	ONVDGLHVRSGFPROKLAEL#GNMFVECAKCKTOYVRDTVVGTMGLTGRLCTVAKARGLAACRGELRDTILDWEDSLPDRDLALADEAS	204	Q8N6T7	SIR6_HUMAN
	167	QNCDGLHLRSGLPRTAISEL <mark>H</mark> GNMYIEVCTSCVPNREYVRVFDVTERTALHRHQTGRTCHKCGTQLRDTIVHFGERGTLGQPLNWEAATEAA	258	Q9NRC8	SIR7_HUMAN
		Catalytic Histidine			
	431	* : :*:* : DEVDLLIVIGSSLKVRPVALIP-SSIPHEVPQILINREPLPHLHFDVELLGDCDVIINELCHRLGGEYAKLCCNPVKLSEITEKPPRTQKE	E 20	Q96EB6	SIR1_HUMAN
	252	DEVOLUTIVESSLOVOPFAS LI-SKAPLSTPRILLINKEKAGGSDFLGMIMGLGGGMDFDSKKAYRDVANLGECONGCLALAELLGMKKEVRRE	348	08IXJ6	SIR1_HUMAN
	310	PMADLLLIGTSLEVEPFASLT-EAVRSSVPRLLINRDLVGPL	390	Q9NTG7	SIR3_HUMAN
	251	KEADSLLVVGSSLQVYSGYRFILTAWEKKLPIAILNIGPTRSDDLACLKLNSRCGELLPLIDPCPC	314	Q9Y6E7	SIR4_HUMAN
	240	AHCDLCLVVGTSSVVYPAAMFAPQVAARGVPVAEFNTETTPATNRFRFHFQGPCGTTLPEALACHENETVSACHENETVS	310	Q9NXA8	SIR5_HUMAN
	205	RNADLSITLGTSLQIRPSGNPLATKRRGGRLVIVNLQPTKHDRHADLRIHGYVDEVMTRLMKHLGLEIPAWDGPRVLERALPPLPRPPTPKLEPKE	301	Q8N6T7	SIR6_HUMAN
	259	SRADTILCLGSSLKVLKKYPRLWCMTKPPSRRPKLYIVNLQWTPKDDWAALKLHGKCDDVMRLLMAELGLEIPAYSRWQDPIFSLATPLRAGEE	352	Q9NRC8	SIR7_HUMAN
	521	LAYLSELPPTPLHVSEDSSSPERTSPPDSSVIVTLLDQAAKSNDDLDVSESKGCME-EKPQEVQTSRNVESIAEQMENPDLKNVGSSTGEKNERTSVAGTVRKCWPNRVAK	630	096EB6	SIR1 HUMAN
	349	HASIDAOSGAGVPNPSTSASPKKSPPPAKDEARTTER-EKPQ	389	Q8IXJ6	SIR2_HUMAN
	391	HASIDAQSGAGVPNPSTSASPKKSPPPAKDEARTTER-EKPQ	399	Q9NTG7	SIR3_HUMAN
	315		314	Q9Y6E7	SIR4_HUMAN
	311 302	ESPTRINGSIPAGPKGEPCAGHNGSEPASPKRERPTSPAPHR	310 355	Q9NXA8 08N6T7	SIR5_HUMAN SIR6_HUMAN
	353		400	Q9NRC8	SIR7 HUMAN
					_
	631	EQISRRLDGNQYLFLPPNRYIFHGAEVYSDSEDDVLSSSSCGSNSDSGTCQSPSLEEPMEDESEIEEFYNGLEDEPDVPERAGGAGFGTDGDDQEAINEAISVKQEVTDMNYPSNKS 74		Q96EB6	SIR1_HUMAN
	390 400			Q8IXJ6 Q9NTG7	SIR2_HUMAN SIR3_HUMAN
	315	31		09Y6E7	SIR4_HUMAN
	311	31		Q9NXA8	SIR5_HUMAN
	356		5	Q8N6T7	SIR6_HUMAN
	401	40	0	Q9NRC8	SIR7_HUMAN

Figure S1 (Related to Figure 1)

- (A) Uniprot sequence alignment of human sirtuins 1-7 using the identifiers: Q96EB6, Q8IXJ6, Q9NTG7, Q9Y6E7, Q9NXA8, Q8N6T7, Q9NRC8 for SIRT1-7, respectively.
- (B) Newick format file representing the tree constructed by FastTree from all sirtuin sequences assigned to the sirtuin PFAM domain (PF02146) longer than 40 amino acids; the names of internal nodes give the support for these nodes, available for download at https://figshare.com/s/e51648137c9a0314616e;
- (C) Tree built from all sirtuin sequences of length greater than 40; this tree supports the classical grouping of the human sirtuins and highlights the existence of several other, mainly microbial sirtuin clusters, available for download at https://figshare.com/s/e51648137c9a0314616e;
- (D) Specificity determining position scores obtained by applying the GroupSim algorithm to an alignment of 81 diverse sirtuins; several positions in the SIRT4 'RQRYWAR' sequence received high scores indicative of importance in distinguishing class II sirtuins from other classes, available for download at https://figshare.com/s/e51648137c9a0314616e.

	SIRT1	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7
SIRT1							
SIRT2	36.94						
SIRT3	35.17	47.16					
SIRT4	<mark>24.46</mark>	26.07	26.98				
SIRT5	26.79	24.81	28.4	28.37			
SIRT6	20.74	25.17	26.85	<mark>28</mark>	20.72		
SIRT7	21.29	20.71	25.09	<mark>26.79</mark>	22.87	37.95	

Figure S2 (Related to Figure 2). Identity matrix of SIRT4 compared to other sirtuins; accessed from: http://www.ebi.ac.uk/Tools/services/rest/clustalo/result/clustalo-I20140522-174546-0317-16067290-es/pim

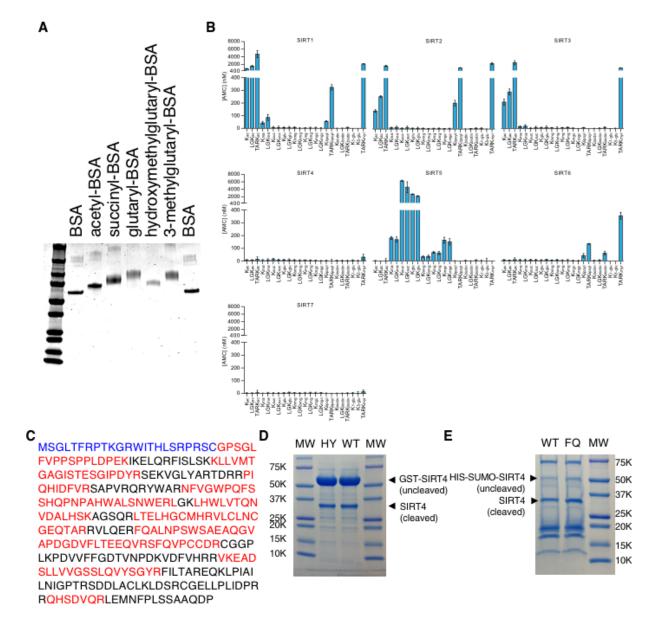


Figure S3 (Related to Figure 3).

- (A) Coomassie-stained gel of BSA incubated with indicated anhydrides or CoA species; gel shift demonstrates successful acylation; 2 µg of each BSA on 4-16% SDS gel;
- (B) Acylated lysine (K), leucine-glycine-lysine (LGK), or threonine-alanine-arginine-lysine (TARK) peptides with a fluorogenic 7-amino-4-methylcoumarin (AMC) probe were incubated with sirtuins SIRT1-SIRT7 obtained from commercial sources. Upon removal of the acyl group and trypsin treatment, the AMC fluorophore is released.
- (C) Mass spectrometry results of mSIRT4 coverage; blue, mitochondrial targeting sequence (MTS); red, peptides identified after tryptic cleavage; raw data is in Supplementary Table 2.
- (D) Representative gel showing recombinant GST-SIRT4 and GST-SIRT4HY fusion proteins were expressed in bacteria. The ~60 kDa GST-SIRT4 and -SIRT4HY fusion proteins (uncleaved) were then incubated with PreScission Protease to cleave off the GST-tag and release the ~33 kDa untagged SIRT4 proteins (cleaved). 4-20% acrylamide gel, 10 µg total protein loaded per lane, Stained with InstantBlue Coomassie stain,
- (E) Representative gel showing recombinant His-SUMO-SIRT4 and His-SUMO-SIRT4FQ fusion proteins were expressed in bacteria. The ~50 kDa His-SUMO-SIRT4 and -SIRT4FQ fusion proteins (uncleaved) were then incubated with SUMO Protease to cleave off the His-SUMO-tag and release the ~33 kDa untagged SIRT4 proteins (cleaved); 4-20% acrylamide gel, 5 µg total protein loaded per lane, Stained with InstantBlue Coomassie stain.

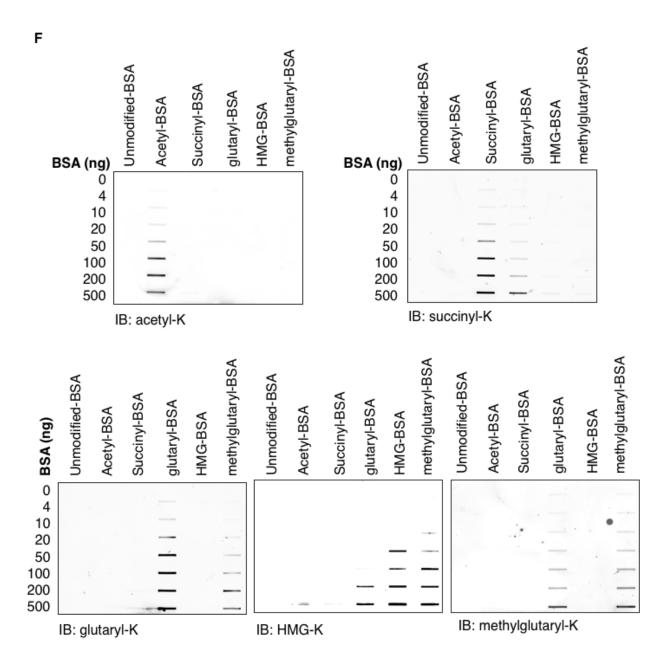


Figure S3 (Related to Figure 3), continued.
(F) Slot blots to evaluate cross-reactivity of acyl-K antibodies. A range of various acyl-BSAs (1-500 ng) were applied to each blot, then probed with indicated acyl-K antibodies.

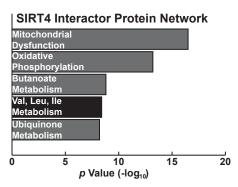


Figure S4 (Related to Figure 4). SIRT4 protein interaction network based on Ingenuity Pathway Analysis from bait-prey co-immunoprecipitation mass spectrometry experiments.

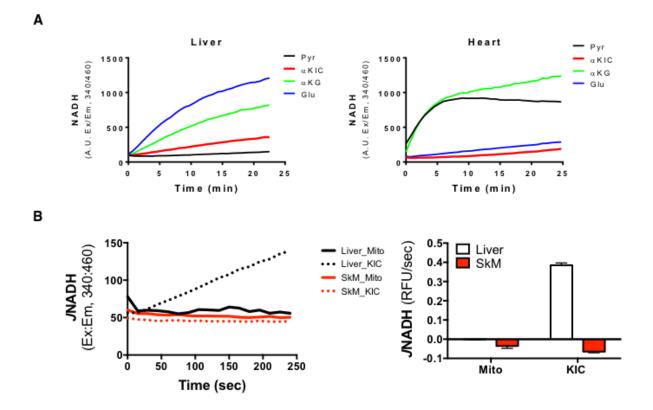


Figure S5 (Related to Figure 5).

- (A) Representative trace of pyruvate (Pyr), α -ketoisocaproate (α KIC), α -ketoglutarate (α KG), and glutamate (Glu) flux measured *ex vivo* in WT and SIRT4KO male mouse liver mitochondria, monitored by NADH fluorescence (excitation 340nm/emission 460nm), representative of n=9/9, WT/SIRT4KO mice.
- (B) Left: representative traces of basal (Mito) and KIC oxidization rates shown by NADH fluorescence in liver and skeletal muscle (SkM) in male wild-type mice. Right: Summary of rate of basal and KIC-stimulated oxidation; n=4/4.

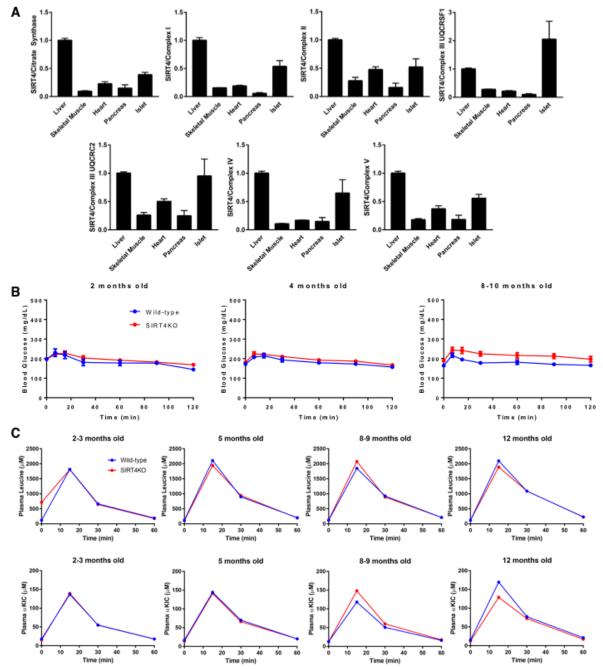


Figure S6 (Related to Figure 6).

(A) SIRT4 expression in liver, skeletal muscle (SKM), heart, pancreas, and pancreatic islets normalized to individual mitochondrial markers; citrate synthase (CS), complex I (CI), complex II (CII), complex III subunit 5, ubiquinol-cytochrome C reductase, Rieske iron-sulfur polypeptide 1 (CIII, UQCRFS1), complex III subunit 2, ubiquinol-cytochrome C reductase core protein II (CIII, UQCRC2), complex IV (CIV), complex V (CV). (B) Blood glucose was measured in 2 month old (n=4/7 WT/SIRT4KO), 4 month old (n=15/15), and 8-10 month old (n=11/11) wild-type and SIRT4KO male mice following an oral gavage of 0.3 mg/g leucine. (C) Plasma leucine and α-ketoisocaproate (αKIC) levels were measured by mass spectrometry in 2-3 month old (n=6/5 WT/SIRT4KO), 5 month old (n=6/4), 8-9 month old (n=5/4), and 12 month old (n=4/7) wild-type and SIRT4KO male mice following an oral gavage of 0.3 mg/g leucine. Plasma samples from each genotype at each time point were pooled to obtain enough plasma for reliable measurements by mass spectrometry.